

Virological and serological study of human infection with swine influenza A H1N1 virus in China[☆]

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ABSTRACT

Background: Pigs are considered to be “mixing vessels” for the emergence of influenza viruses with pandemic potential. 2009 Pandemic Influenza H1N1 further proved this hypothesis, and raised the needs for risk assessment of human cases caused by swine influenza virus.

Methods: A field investigation was conducted after a case identified with infection of European avian-like swine influenza H1N1 virus. The diagnosis was confirmed by real-time PCR, virus isolation, whole genome sequencing and serological assays. Samples from local pigs and close contacts were tested to identify the source of infection and route of transmission.

Results: The virus from the index case was similar to viruses circulating in the local pigs. The case's grandfather was asymptomatic with sero-conversion. A total of 42.8% of swine sera were positive for European avian-like swine H1N1.

Conclusions: This study highlighted the importance of performing surveillance on swine influenza to monitor new virus emergence in humans.

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Introduction

Three subtypes of influenza A viruses are currently circulating among pigs throughout the world, including classic swine H1N1, avian-like H1N1, human-like or avian-like H3N2, reassortant H3N2 and various genotype H1N2 viruses (Brown, 2000; Nardelli et al., 1978; Webster et al., 1992). Zoonotic infections with swine influenza A viruses have been reported sporadically in the USA and Europe since 1974 (de Jong et al., 1988; Myers et al., 2007; Thompson et al., 1976; Top and Russell, 1977). Avian-like swine H1N1 influenza virus was firstly reported in Europe in 1976 (Scholtissek et al., 1983). Human infections with avian-like swine H1N1 have been sporadically reported in Europe (Gregory et al., 2003; Rimmelzwaan et al., 2001; Shinde et al., 2009). Various swine

influenza viruses have been detected in China, including classic swine influenza virus (Guo et al., 2000; Shortridge and Webster, 1979), avian-origin H1N1 (Guan et al., 1996), European avian-like H1N1 (Liu et al., 2009; Vijaykrishna et al., 2011), H3N2 subtypes (Yu et al., 2008), reassortant H1N1 and H3N2 subtypes, and occasionally avian H5N1 and H9N2 (Li et al., 2004; Liu et al., 2011; Yu et al., 2011). However, human infections with swine influenza virus have rarely been documented in China. Only one child was recorded as infected with swine influenza H3N2 virus in Hong Kong during 1999 (Gregory et al., 2001). Swine have been considered to be an intermediate host for potential pandemic influenza viruses, and the 2009 Pandemic H1N1(H1N1 pdm09) further highlighted the importance of swine influenza surveillance as a part of pandemic preparedness (Garten et al., 2009). Therefore, it is essential to conduct comprehensive investigations after human infections with swine influenza virus occurred.

In this study, we report a human case infection with European avian-like swine H1N1 influenza virus in Jiangsu Province of PR China in 2010. A field investigation was conducted to identify the infection source and assess the potential risks for human infection. The virological and serological results suggested that local pigs were source of human infection, and limited human cases in this region were tested serologic positives.

[☆]We conducted a field investigation after a human case of European avian-like swine influenza virus H1N1 infection in China was detected. The virus isolated from this case was similar to viruses isolated from local pigs, and a case of subclinical infection was also detected.

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Results

Index case confirmation

The case's tracheal specimens collected on days 4 and 5 were positive for the influenza A/M gene but negative for specific rRT-PCR primers and probes of human influenza (H1, H3, H1N1 pdm09) and avian influenza (H5, H7 and H9). The specimens were tested by the Nanjing and Jiangsu Centers for Disease Control and Prevention (CDC), and the results were further confirmed by National Institute for Viral Disease Control and Prevention, China CDC. The consecutive tracheal specimens collected on day 10 and days 16–24 (except for day 21) after the patient's onset of illness were positive for the influenza A/M gene, indicating a long period of virus shedding. The tracheal specimens collected on days 4 and 5 were inoculated into SPF embryonated chicken eggs and MDCK cells. The isolates were obtained from both samples and designated as A/Jiangsu/1/2011 (JS1). The genome sequences of the original samples and the virus isolate were similar to the European avian-like swine H1N1 viruses (EA H1N1) and different from seasonal H1N1 and H1N1 pdm09 (Fig. 2). Paired sera from the index case indicated sero-conversion, with a greater than 4-fold increase in the HI antibody against JS1 and a 4-fold increase from < 10 to 40 against A/California/07/2009 (CA07) (Table 1). Cross-reactivity was observed between JS1 and H1N1 pdm09, which was also supported by the antigenic characterization results using ferret antisera (Table 3).

Investigation of the infection source from the index case's farm

The poultry and pigs were raised freely in the index case's back yard. The chickens were ill and dead about 8 days before the case's onset of illness. 12 of 26 pigs had been ill with fatigue 10 days before the case's onset, and 2 pigs that had been live while others were slaughtered. A total of 43 samples from throat and cloacal swabs and feces from chicken, geese and ducks and 12 tissue specimens from dead chicken were tested negative for influenza A/M/H5/H9 by rRT-PCR and virus isolation, but Newcastle disease viruses were isolated from 3 of 12 organs. The sera samples of 3 geese and 5 chickens from the case's back yard were negative to JS1 by HI tests.

Ten nasal swabs and 2 serum samples were collected from pigs of the subject's family and his neighbors. No virus isolates were obtained from these nasal swabs, after cultured in SPF eggs and MDCK cells for 3 passages, but two pig serum samples had an HI antibody titer of 40 against JS1 virus.

Investigation of swine samples from the local slaughterhouses

To investigate the influenza virus circulating in local swine and to assess the risk of human infection, 21 blood samples and 60 lung tissue samples of pigs were collected from 3 local slaughterhouses in the county of the human case (Fig. 1). Six of 30 lung tissue samples collected during January 5–8 tested positive for influenza A/M by rRT-PCR, and 4 isolates were obtained, while all 30 samples collected during March 7–10 were negative for influenza A/M. These 4 isolates were designated as A/Swine/Jiangsu/S14/2011 (S14), A/Swine/Jiangsu/S15/2011 (S15), A/Swine/Jiangsu/S16/2011 (S16) and A/Swine/Jiangsu/S30/2011 (S30); these isolates were highly related to JS1, with similarities of 96.0–99.7% among the eight genome sequence (Table S2). They clustered with European avian-like H1N1 swine influenza viruses isolated in Jiangsu recently in phylogenetic trees of 8 segments (Figs. 2 and S1). The amino acid analysis of the HA1 domain found that all of the antigenic sites of the 4 isolates are identical with that of JS1 (Table S3). There were no mutations at positions 119E, 152R, 275H

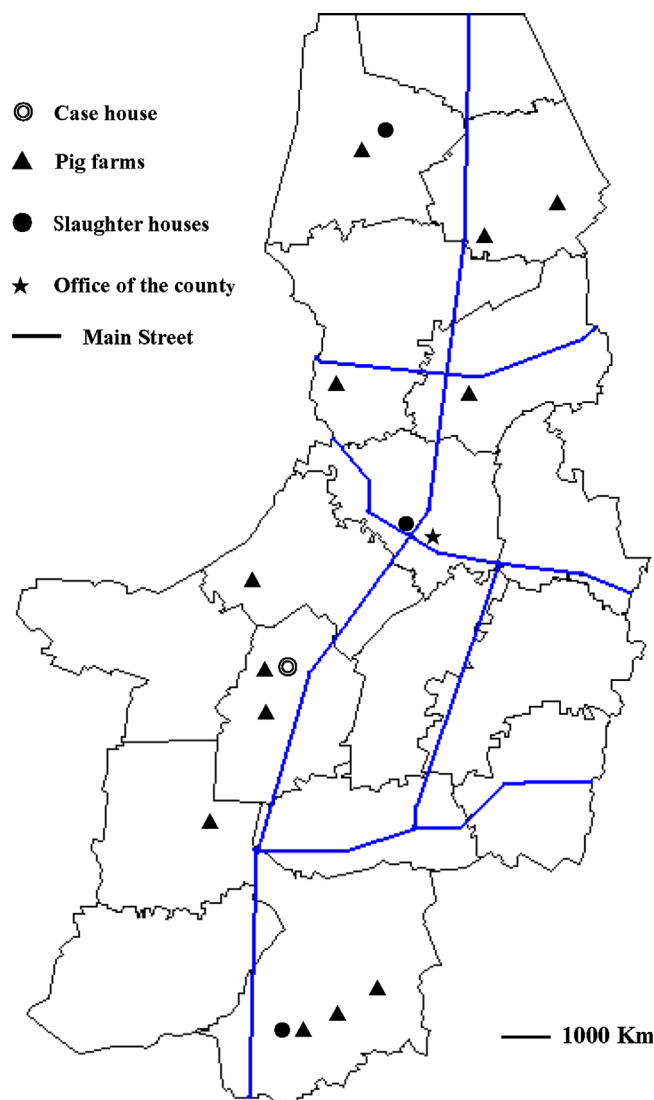


Fig. 1. Map of the local slaughterhouses from which swine samples were collected.

and 295N of the NA protein, indicating that the isolate may be sensitive to neuraminidase inhibitor drugs which were used in the case treatment. The M2 proteins possessed the mutation 31N, which accounts for the resistance to amantadine.

Totally 9 of 21 (42.8%) pig sera were of HI titer ≥ 40 for JS1, while 6 of 21 (28.6%) were of HI titer ≥ 40 for S16. In addition, 11 of 21 (52.4%) pig sera were of HI titer ≥ 40 for CA07, and 3 of 21 (14.2%) were of HI titer ≥ 40 for the classic swine H1N1A/Swine/Shanghai/1/2005 (Table 2). The pig sera with antibody titer of ≥ 40 against 4H1N1 viruses were shown in the Venn diagram (Fig. S2). Some of the sera reacted with different viruses were shown in the cross parts of the circles.

Antigenic characterization. A panel of ferret sera was used for antigenic characterization, which included ferret sera against human seasonal influenza virus H1N1 A/Brisbane/59/2007 and A/Solomon Islands/03/2006, H1N1 pdm09 A/California/7/2009 (CA07), classical swine influenza virus A/New Jersey/8/1976 and A/Swine/Beijing/156/1991, and European avian-like influenza virus JS1 and S16. The results showed that human isolate JS1 was antigenically similar to swine isolate S16. Both isolates reacted well with the ferret antisera of H1N1 pdm09 and classic swine H1N1, while there was no cross reaction with human seasonal

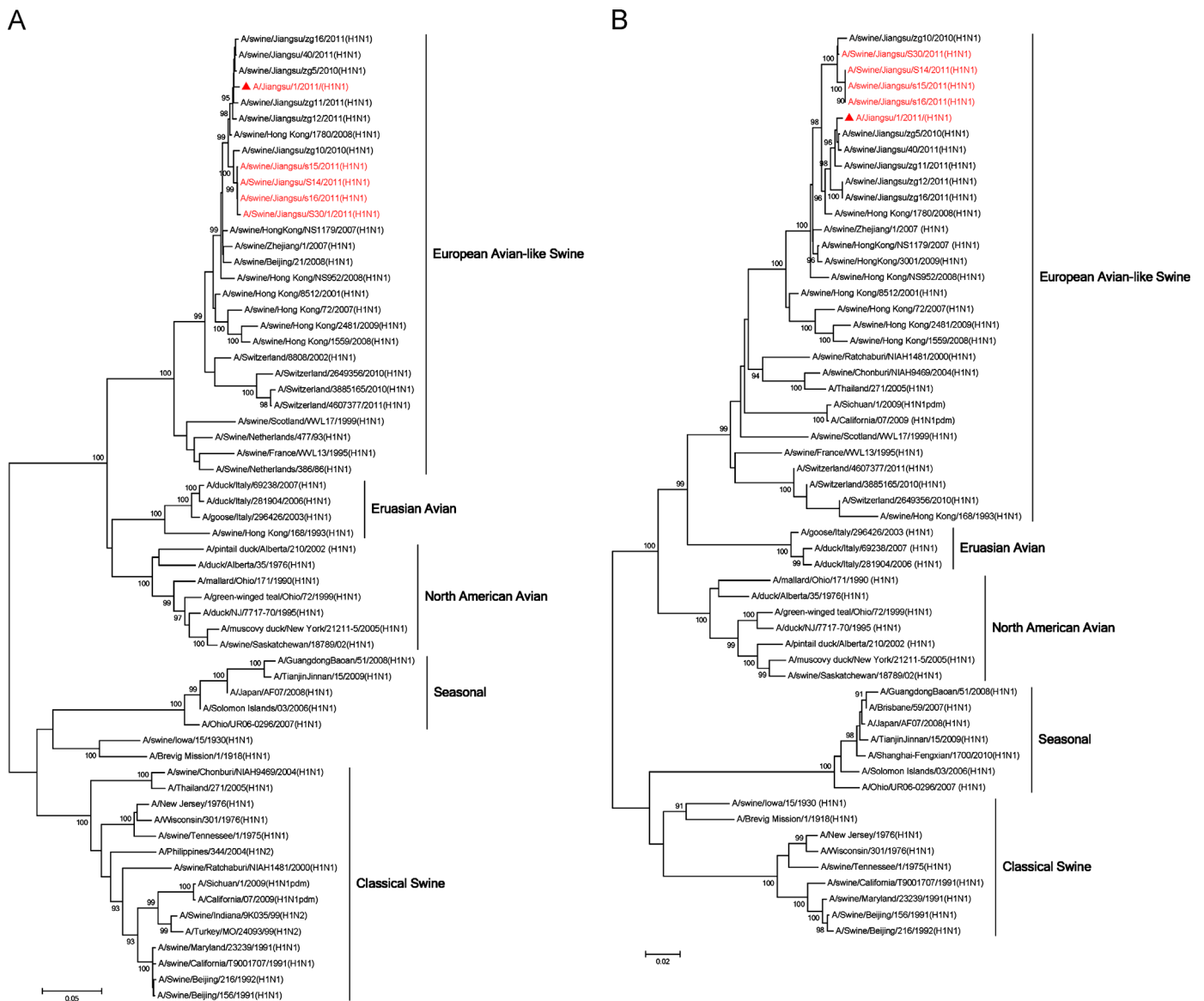


Fig. 2. Phylogenetic trees of the HA (A) and NA (B) genes of the H1N1 influenza A viruses. The trees were constructed based on the open reading frames of the HA and NA genes using the neighbor-joining method of MEGA 5.0, and 1,000 bootstrap trials were used to assign confidence values to the groupings. Only the bootstrap values of 90% are shown. The scale bars represent substitutions per site. Viruses from swine isolated from the local slaughterhouses are red, and the virus of the index case is red with a triangle.

Table 1

The positive HI antibody titers to representative influenza A H1N1 viruses in serum samples: the index case and the residents with HI antibody titer to JS1 of ≥ 40 .

Relationship	Age (years)	Sex	Collection date	HI titers against			Swine exposure
				A/Brisbane/59/2007	A/California/07/2009	A/Jiangsu/1/2011	
Case	3 years and 8 months	Male	3 Jan 2011 31 Jan 2011	80 160	< 10 40	< 10 160	No direct contact
Grandfather	54	Male	5 Jan 2011 31 Jan 2011	< 10 160	20 40	< 10 1280	Raised pigs
Great-grandmother	84	Female	5 Jan 2011 26 Jan 2011	20 < 10	< 10 < 10	40 20	Pigs raised on own farm
Same village (8A)	39	Male	9 Jan 2011	10	< 10	40	No pigs raised on own farm
Same village (78A)	67	Female	14 Jan 2011	20	40	40	

H1N1 (Table 3). Similarly, the H1N1 pdm09 and classic swine viruses reacted with ferret sera of both Jiangsu viruses at high titers.

Avian-like swine H1N1 isolated in Hong Kong and human isolates from Switzerland and Netherlands were compared with JS1 at the antigenic site of the HA1 protein. The amino acids in the

Table 2
HI antibody titers of pig sera to influenza A H1N1 viruses.

Sera from	No. of sera	No. of sera with HI titers ≥ 40 against			
		pdm09 H1N1 A/California/07/2009	Case isolate A/Jiangsu/1/2011	Swine isolate A/Swine/Jiangsu/S16/2011	Classic swine H1N1 A/Swine/Shanghai/1/2005
Pigs of index case's neighbor	2	0	2 (100%)	1 (50%)	1 (50%)
Pigs of slaughter houses	21	11 (52.4%)	9 (42.8%)	6 (28.6%)	3 (14.2%)

Table 3
Antigenic characterization of different influenza A H1N1 viruses by HI assays

Test antigens	Ferret sera against						
		BR/59	CA/07	NJ/8	SW/BJ/156	JS/1/2011	SW/JS/S16
Seasonal H1N1	A/Solomon Islands/03/2006		320	< 10	< 10	< 10	< 10
	A/Brisbane/59/2007		640	< 10	< 10	< 10	< 10
pdm09 H1N1	A/California/07/2009		< 10	1280	320	640	640
	A/Sichuan/1/2009		< 10	1280	320	640	640
Classic swine H1N1	A/New Jersey/8/1976		< 10	640	640	320	320
	A/Swine/Beijing/156/1991		< 10	640	320	1280	320
European avian-like swine H1N1	A/Jiangsu/1/2011		< 10	1280	160	320	640
	A/Swine/Jiangsu/S16/2011		< 10	160	80	160	640
	A/Swine/Jiangsu/S14/2011		< 10	2560	320	1280	2560
	A/Swine/Jiangsu/S15/2011		< 10	2560	320	1280	2560

Note: The virus titer against the same lineage of H1N1 is shown in bold. Underlined values represent homologous antibody titers.

Abbreviations: A/Brisbane/59/2007, BR/59/2007; A/California/07/2009, CA/07/2009; A/New Jersey/8/1976, NJ/8/1976; A/Swine/Beijing/156/1991, SW/BJ/156/1991; A/Jiangsu/1/2011, JS/1/2011; A/Swine/Jiangsu/S16/2011, SW/JS/S16/2011.

Table 4
The seroprevalence of people in different groups.

Groups	Acute sera (No. of HI ≥ 40 to JS1)	Convalescent sera (No. of HI ≥ 40 to JS1)
Family members	5 (1)	5 (1)
Neighbors	14 (0)	12 (0)
Health care workers	16 (0)	16 (0)
Same villagers	60 (2)	36 (0)
Neighboring villagers	18 (0)	11 (0)
Total	113 (3)	80 (1)

HA1 antigenic sites (H1 numbering) of JS1 were similar to the slaughterhouse viruses in our study, and to the early EA representative virus A/swine/Hong Kong/8512/2001 but were different from the new variant EA swine H1N1 virus A/swine/Hong Kong/1559/2008 at eight residues (Vijaykrishna et al., 2011). There were two amino acid differences in JS1 HA1 at positions 3 and 271 compared to the Swiss strains from 2010 and 2011, and five and seven different amino acids compared with the Netherlands 1993 and 1986 strains from humans (Table S3).

Sera prevalence investigation of close contacts and residents. The influenza-like illness rate in local hospitals was normal before and 1 month after the onset of case. None of the close contacts, including family members, health care workers and other close contacts, reported ill within 2 weeks after the index case was detected. 16 nasal and throat swabs of 13 close contacts all tested negative for influenza virus by rRT-PCR and virus isolation.

13 of 113 acute sera samples and 1 of 80 convalescent sera samples of close contacts were HI antibody-positive against JS1 using HI titer of 40 as a cutoff (Tables 1 and 4). Two positive acute sera samples were collected from the residents of the local

villages, one of which had HI antibody titers to CA07 at 40, but no convalescent sera was obtained from these two subjects. The subject, aged 39, was considered to be sero-positive with an HI titer of 40 to JS1 but was negative to CA07. The third acute serum sample was collected from the index case's 83-year-old great-grandmother, but the HI antibody of her second serum sample was lower than 40, and it could not exclude a cross-reaction with other seasonal H1N1 influenza viruses. Interestingly, the HI titers to JS1 of the index case's grandfather's serum sample increased more than 4-fold greater (from < 10 to 640), and HI titers to A/Brisbane/59/2007 increased from < 10 to 160 simultaneously (Table 1). According to the questionnaire, he did not exhibit any influenza-like illness symptoms from 2 weeks before and one month after the index case's onset, but he had frequent exposure to the family's pigs because he was involved in raising pigs.

Discussion

European avian-like swine H1N1 virus was first introduced into European pigs in 1979, and the isolation rate from pigs has increased in China since 2001 (Liu et al., 2009; Shortridge and Webster, 1979; Vijaykrishna et al., 2011). Sporadic human infections by this virus lineage have been reported in Netherlands and Switzerland. The symptoms were indistinguishable from other human influenza infections (Gregory et al., 2003; Myers et al., 2007; Rimmelzwaan et al., 2001). In this study, we reported a human case of European avian-like swine influenza H1N1 virus infection with severe pneumonia, followed by coma and eventual death. One potential factor for boy's death may relate to his history of steroid treatment.

The index case's samples were tested by rRT-PCR, and the HA primers and probes of human seasonal H1N1 and H1N1 pdm09 could not detect this EA swine H1N1 virus. The isolate could be amplified by conserved sequencing primers of H1N1 viruses.

So the case infected with EA swine virus was confirmed by sequence analysis. Specific nucleotide diagnostic reagents should be developed for diagnosis.

To identify the source of infection and the potential human-to-human transmission, a field investigation was conducted after the index case was reported. The virus that was isolated from the index case was quite similar to viruses isolated from local pigs, although we were unable to isolate the virus from 2 pigs that remained in his farm, but 2 sera from two pigs were of titer > 40 to JS1. However, a recent report showed that the virus isolated from the pigs from this case backyard strongly indicated that the human infection originated from the pigs (Yang et al., 2012).

Although the genetic analysis found that the HA proteins of the JS1 were different from those of classic swine H1N1, H1N1 pdm09 pandemic H1N1 and seasonal H1N1 influenza viruses, the antigenic characteristics of JS1 revealed that it is close to the avian-like swine H1N1 as well as classic swine H1N1 and H1N1 pdm09 viruses. These results are comparable with the antigenic analysis results reported from Hong Kong, in that ferret sera of early Eurasian swine H1N1 viruses (2001–03) cross-reacted with classic swine and H1N1 pdm09 viruses of swine isolates but that the new variants were different with them (Vijaykrishna et al., 2011). And the amino acids similarity of JS1 and SW/HK/8512/2001 in the antigenic sites were the basis of the HI antigenic responses (Table S3).

The seroprevalence investigation results confirmed that the index case's grandfather presented seroconversion without any influenza-like illness symptoms, suggesting that he experienced an asymptomatic infection. Some similar cases have been reported previously (Myers et al., 2007; Yang et al., 2012). For his frequent exposure to pigs, we were unable to conclude a human-to-human transmission, even though he had close contact with the index case. Three sera were HI antibody-positive, including the serum sample from the index case's great-grandmother and 2 residents from the same village, 2 of which exhibited negative HI titer to CA07. The H1N1 pdm09 viruses can react well with ferret antisera raised to European avian-like H1N1 viruses in our antigenic analysis, and one study of human sera infected with pandemic H1N1 virus also found cross-reactivity (Perera et al., 2011), while the cross reactivity between seasonal H1N1 and the new isolate should be further explored by testing a panel of historic seasonal H1N1 of epidemiologic significance. One person considered serum-positive also exhibited titers to CA07. All health care worker contacts were sero-negative for JS1. This result indicated that direct contact with pigs or common environments of exposure were the risk factors for human infection with swine influenza virus, and there was no evidence for human-to-human transmission.

The sero-prevalence investigation results of local pigs suggested that the incidences of European avian-like swine H1N1 and H1N1pdm09 infection were high in local pigs, at approximately 40% and 50%, respectively. A report of swine surveillance from Jiangsu is consistent with our findings, with both types of virus isolated (Zhao et al., 2012). Different reassortants between H1N1 pdm09 and other swine influenza viruses have been reported since 2009 (Tremblay et al., 2011; Zhu et al., 2011). Of particular note is the recent report from the US CDC about a triple swine H3N2 influenza virus that contained the M gene from pandemic H1N1, which has caused more than 300 cases in the USA with limited human-to-human transmission (Centers for Disease Control and Prevention, 2012a, 2012b).

Conclusion

Pigs have served as intermediate hosts for influenza virus with pandemic potential, such as the H1N1 pdm09 virus (Smith et al.,

2009). Our study indicated that the swine virus continues to infect humans sporadically without reassortment. This type of human infection with swine influenza may occur sporadically, and its transmissibility should be assessed when human case or clusters occur. Both virology and serology study in humans and swine are important. Our study highlighted the importance of influenza surveillance in swine and human population for pandemic preparedness.

Note: The genomic sequences of the isolates are available in GISAID and GenBank under accession numbers.

Materials and methods

Index case investigation

On 31 December 2010, a 3-year, 8-month-old boy from a county of Jiangsu Province developed cough and rhinitis. He was admitted to the local hospital with dyspnea and fever and recovered with mechanical ventilation, but he fell into coma on 2 January, 2011. Bilateral patchy infiltrates were observed by chest radiography (Zhao et al., 2011). The patient had a history of renal disease and had undergone steroid treatment for two months. He experienced brain death during medical care and died after 40 days. He lived in a farm with cows, pigs, chickens, geese and ducks. Some of the pigs and chickens were sick two weeks before his onset.

Sample collections

Tracheal aspirate specimens were collected from the index case on January 4, 5, and 10, 2011 for diagnosis. Subsequent tracheal samples were taken from January 16 to 24, with the exception of January 21, and used to guide the treatment of antiviral drug oseltamivir. Serum samples were collected on day 3 and day 31 after illness onset.

To identify infection sources, animal samples were obtained from boy's family back yard farm and local slaughterhouses. Total 43 poultry samples were collected from January 5 through 7 in the back yard, including 13 fecal samples and 18 cloacal and throat swabs from live chickens and 12 tissue specimens from 3 dead chickens. Moreover, 8 sera specimens of geese and chicken were taken. 10 nasal swabs and 2 sera samples were collected from pigs from boy's family and his neighbors from January 8 through 9. In addition, 21 blood and 30 lung samples were collected from pigs during January 5–18, and additional 30 pig lung specimens were collected during March 7 to 10 from three local slaughterhouses (Fig. 1).

To investigate the potential transmission and subclinical infections among close contacts and in the community, respiratory and serum samples from close contacts and residents in the same and neighboring villages were collected. A total of 16 nasal and throat swabs were collected from 13 close contacts from March 3 through 7. A total of 113 serum samples collected from close contacts and local residents from 5–14 January (within 14 days after the index case's illness onset) were defined as acute sera. 80 of 113 serum samples were available and collected on 26 and 31 January as convalescent sera. Detailed information from humans and animal samples are shown in Table S1. Feces, respiratory swabs and tissue samples were maintained in Eagle's medium with 0.5% bovine serum albumin (BSA) and antibiotics and stored at -70°C (WHO, 2002). The serum samples were centrifuged and stored at -20°C .

A questionnaire survey was conducted to record all human subjects involved, and collected history of animal exposure, history of contact with the index case, and influenza-like illness symptoms within one month. This investigation was performed

according to the National Pandemic Preparedness and Response Plan, and thus, the subject institution review was waived.

Real-time PCR testing and virus isolation

Respiratory swabs, tissue samples and feces were tested using real-time reverse transcription PCR (rRT-PCR), 10-day-old specific pathogen-free (SPF) chicken embryonated eggs, Madin Darby Canine Kidney (MDCK) cells and sequencing. RNA extraction was performed using a QIAamp Virus RNA Mini Kit (Qiagen, Shanghai, China). RT-PCR assays were performed using the Ag Path-ID TM one-step RT-PCR kit (AB, Shanghai, China). Primers and probes were used for influenza A/M, seasonal H1, H3, H1N1 pdm09 and avian influenza (H5, H7 and H9) (WHO, 2007, 2009). The samples were inoculated into cells and eggs. The eggs were incubated at 35 °C for 72 h, and the cells were cultured at 35 °C for 6 days or collected after cytopathogenic effects were observed. Fluid harvested was tested using hemagglutination assays and rRT-PCR. Three passages were attempted with the HA-negative samples.

Genome sequences analysis

The genomes of isolates were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (AB) and an AB 3730XL analyzer. Editing and analysis of the sequences were performed with Lasergene. The alignment of sequences was performed with ClustalW, and Mega 5.0 was used to generate phylogenetic trees with 1000 replication bootstrap values (Tamura et al., 2011). Representative sequences of H1 subtype influenza viruses, including North American avian, Eurasian avian, seasonal H1N1, classical swine and European avian-like swine influenza sequences, were used for phylogenetic tree analysis.

Hemagglutination inhibition (HI) test

The standard HI test was conducted using 0.5% turkey erythrocytes (WHO, 2002). Sera were treated with receptor-destroying enzymes overnight and tested with 2-fold serial dilutions starting from 1:10. HI titers were defined as the reciprocal of the highest dilution of sera that completely inhibited hemagglutination. Ferret sera were used for antigenic analysis. Fourfold or greater increases in paired sera of humans were considered to be related to infections. A single serum was considered to be positive if the HI titer of ≥ 40 and no HI antibody titers positive to any other different H1N1 viruses tested. An HI titer of ≥ 40 is associated with 50% risk reduction of influenza infection in human population (Hobson et al., 1972). The viruses used in the antigenic analysis and the human and swine sera tests are listed in (Tables 1–3).

Author contributions

Y.S. and W.H. directed the study, R.Z., C.X. and Z.X. designed the field investigation, and X.H. and N.X. coordinated the field investigation. L.D., X.Q., D.W., S.M., T.B., X.L., X.Z., S.Y. and Z.L. performed the experiments. R.Z., L.D. and M.L. performed data analysis. Y.S. and L.D. wrote the paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2013.07.022>.

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